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A Highly Divergent 33 kDa *Cryptosporidium parvum* Antigen

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ABSTRACT: Previous studies comparing the genome sequences of *Cryptosporidium parvum* with *Cryptosporidium hominis* identified a number of highly divergent genes that might reflect positive selection for host specificity. In the present study, the *C. parvum* DNA sequence cgd8-5370, which encodes a protein whose amino acid sequence differs appreciably from its homologue in *C. hominis*, was cloned by PCR and expressed as a recombinant protein in *Escherichia coli*. Antisera raised against the recombinant cgd8-5370 antigen strongly recognized a unique 33 kDa protein in immunoblots from reducing and non-reducing SDS-PAGE of native *C. parvum* protein. However, anti-Cp33 sera did not recognize the native 33 kDa homologue in *C. hominis*. In an immunofluorescence assay (IFA), anti-Cp33 serum recognized an antigen in the anterior end of air-dried *C. parvum* sporozoites but failed to bind at any sites in *C. hominis* sporozoites, indicating its specificity for *C. parvum*. IFA staining of live *C. parvum* sporozoites with anti-Cp33 serum failed to bind to the parasite, indicating that the CP33 antigen is not on the sporozoite surface, which is consistent with topology predictions based on the encoded amino acid sequence. RT-PCR analysis of cgd8-5370 mRNA before or during *C. parvum* oocyst excystation revealed transcripts only in excysting sporozoites. Thus, Cp33 represents one of a small number of proteins shown to differentiate *C. parvum* from *C. hominis* sporozoites and oocysts.

Cryptosporidiosis is an enteric parasitic disease of humans and animals caused by *Cryptosporidium* species that has a fecal-oral route of transmission (for review see Fayer, 2004; Collinet-Adler and Ward, 2010). Outbreaks of cryptosporidiosis in humans generally arise from contamination of drinking water by *Cryptosporidium hominis* or *Cryptosporidium parvum* oocysts, 2 species that can be differentiated by various genotyping methods (Tanriverdi and Widmer, 2006; Widmer and Lee, 2010; Robinson and Chalmers, 2012). Whereas *C. hominis* has been found restricted to humans, a number of *C. parvum* subtypes have been found to be either zoonotic or anthroponotic (Widmer and Sullivan, 2012). Comparison of the *C. hominis* and *C. parvum* genomes has identified highly divergent protein-coding gene sequences (Sturbaum et al., 2003; Ge et al., 2008; Widmer et al., 2012; Bouzid et al., 2013). In general, these sequences code for proteins of unknown function, but some are hypothesized to be involved in host specificity. The complete sequencing of the *C. parvum* (Abrahamsen et al., 2004) and *C. hominis* genomes (Xu et al., 2004) 10 yr ago has led to insight in the molecular and biochemical nature of these parasites (Rider and Zhu, 2010; Mauzy et al., 2012; Zhang et al., 2012). The genetic determinants of phenotypic differences between *C. parvum* and *C. hominis*, primarily the host range, are still unknown. With the goal of assessing the function of one of several highly divergent genes, the cgd8-5370 gene sequence, which was originally identified by comparing the orthologous protein-coding sequences of *C. parvum* and *C. hominis* (Ge et al., 2008), was expressed as a recombinant antigen in *Escherichia coli*. Antisera prepared against the encoded protein recognized *C. parvum* sporozoites but failed to recognize *C. hominis* sporozoites in immunofluorescence and immunoblotting assays, providing additional evidence for phenotypic differences between the 2 *Cryptosporidium* species.

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Cryptosporidium parvum (Iowa isolate; Abrahamsen et al., 2004) oocysts were purchased from the University of Arizona, stored at 4 C in PBS containing 100 U/ml penicillin, 100 μg/ml gentamicin, and 0.01% Tween 20, and used within 3 mo of propagation in calves. Sporozoites were excysted by suspending the oocysts in 20% bleach and incubating on ice for 10 min. The oocysts were washed twice in cold PBS and then resuspended in growth medium (RPMI with L-glutamine, 10% FBS, 50 mM glucose, 15 mM HEPES, 35 μg/ml ascorbic acid, 1 mM sodium pyruvate, and 0.01% gentamicin) containing 0.4% sodium taurocholate (Sigma Chemical Co., St. Louis, Missouri). The excysted sporozoites (and any remaining intact or empty oocysts) were then dried on multi-well glass slides, immersed for 5 min in cold methanol, allowed to air dry, and stored at −70 C.

RNA was extracted from *C. parvum* oocysts before or during excystation using an RNeasy Mini-Kit (Qiagen, Valencia, California) and described procedures (Jenkins et al., 2011). Contaminating DNA was removed using an in-column DNase step following the manufacturer's procedures (Qiagen). RNA concentrations were estimated using a RiboGreen RNA assay kit (Invitrogen, Carlsbad, California). RT-PCR of RNA (10 ηg) isolated from resting or excysting *C. parvum* oocysts was performed using primers directed to the Cp33 sequence (Cp33 F2- 5′ CAGACTTTACC-TAACGTAGACG 3′, Cp33 R2-5′ TTGGTTGGTCCCATGTCTAC 3′) and a Superscript III reverse transcriptase kit (Invitrogen). RT-PCR was performed in a BioRad Thermocycler using the following conditions: RT 47 C, 30 min, denaturation at 94 C, 3 min, followed by 35 cycles 94 C, 30 sec, 55 C, 30 sec, 72 C, 1 min, and final extension at 72 C, 5 min. The RT-PCR products were analyzed by polyacrylamide gel electrophoresis, followed by EtBr staining, and visualization and capture on a GelLogic 200 Imaging System (Kodak, Rochester, New York).

Cryptosporidium parvum oocysts were frozen-thawed 3 times between a dry ice-ethanol bath and a 37 C water bath in AE buffer (Qiagen). DNA was extracted using a Qiamp DNA Mini-Kit (Qiagen) following methods provided by the manufacturer. DNA concentration and purity was estimated by measuring absorbance at 260 and 280 nm using a NanoDrop ND1000 Spectrophotometer (Nanodrop Technologies, Wilmington, Delaware). The Cp33 coding sequence was amplified using the following primers: Cp33F, 5′ CCGAGCTCATGCTTTTGAATAAG 3′ (underlined = SacI site) and Cp33R, 5′ AGACTGCAGCTACTCAGTACTTTCAC 3′ (underlined = PstI site) and the following PCR conditions: denaturation at 94 C, 3 min, followed by 35 cycles 94 C, 30 sec, 50 C, 30 sec, 72 C, 1 min, and final extension at 72 C, 5 min. The PCR products were analyzed by polyacrylamide gel electrophoresis, followed by EtBr staining, with capture and visualization on a GelLogic 200 Imaging System (Kodak). PCR products were excised from the polyacrylamide gel and eluted overnight from the gel slice in 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 M EDTA, and 0.1% sodium dodecylsulfate (Sambrook et al., 1989) at 37 C. The eluted DNA was precipitated with 2 volumes 100% ethanol, centrifuged for 30 min at 10,000 g at 4 C, washed once with cold 70% ethanol, air-dried, and suspended in 10 ul 1 mM Tris pH 8.0, 0.1 mM EDTA. The Cp33 amplification product and pBADa expression vector (Invitrogen) were digested with SacI and PstI (New England Biolabs, Ipswich, Maine) for 2 hr at 37 C, electrophoresed on a 0.8% agarose gel, followed by EtBr staining, and capture and visualization on a GelLogic 200 Imaging System (Kodak). The SacI-PstI

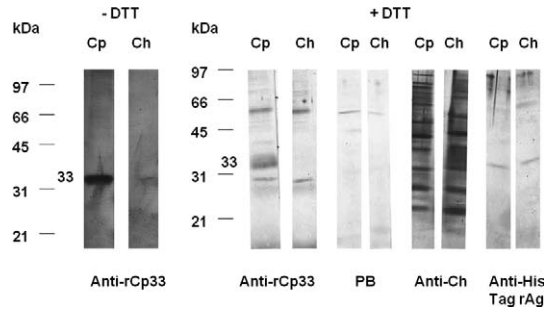

FIGURE 1. Immunoblotting of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts/sporozoite protein with antisera specific for recombinant Cp33. Cp, native *C. parvum* oocysts/sporozoite protein; Ch, native *C. hominis* oocysts/sporozoite protein; -DTT, non-reducing (without DTT); +DTT, reducing (with DTT). Anti-rCP33, antisera specific for rCP33; PB, pre-immunization sera; anti-Ch, antisera specific for total *C. hominis* oocysts/sporozoite protein; anti-HisTag rAg, antisera-specific for an irrelevant HisTag recombinant protein.

restriction-digested Cp33 product and pBADa vector were isolated from agarose using a Qiamp Gel Purification kit (Qiagen), precipitated with ethanol, air-dried, suspended in sterile H_2O, and ligated together overnight at 15 C using T4 DNA ligase (NEB). The ligation mixtures were introduced into *Escherichia coli* DH5 cells using a standard transformation procedure (Hanahan, 1983). Colonies appearing on LB-ampicillin (Amp) plates were cultured overnight in SuperBroth-Amp (Sambrook et al., 1989), and the cultures were processed for plasmid DNA using a Plasmid DNA Mini-Kit (Qiagen). Plasmids harboring Cp33 were confirmed by SacI-PstI digestion and DNA sequencing using pBad-specific primers.

Recombinant pBADa-Cp33 plasmid DNA was introduced into *E. coli* Top10 using standard transformation procedures (Hanahan, 1983). Pilot expression experiments were conducted, which indicated that optimal expression of recombinant Cp33 (rCp33) occurred by induction with 0.2% arabinose (Sigma) for 4 hr at 37 C. Recombinant Cp33 was highly insoluble. Solubilizing required extraction with native binding buffer (20 mM sodium phosphate, 500 mM NaCl pH7.8), followed by denaturing binding buffer (8 M urea, 20 mM sodium phosphate, 500 mM NaCl pH7.8), followed by 0.1% SDS. Solubilized Cp33 was dialyzed overnight against denaturing binding buffer, and then purified by NiNTA affinity chromatography using procedures recommended by the manufacturer (Invitrogen). Isolation and purity of rCp33 was checked by SDS-PAGE and both Coomassie Blue staining and immunoblotting with mouse anti-His antiserum (Invitrogen). Peak eluates from NiNTA purification were dialyzed overnight against PBS, and then reduced to 100 µl volume using an Amicon Ultra-4 concentrator (Merck Millipore, Tullagreen, Ireland). Polyclonal antisera were prepared against rCp33 by a commercial company (Pacific Immunology, Ramona, California) by immunizing 2 rabbits over the course of 2 mo with the primary immunization in Complete Freunds Adjuvant, and 3 booster immunizations in Incomplete Freunds Adjuvant.

Cryptosporidium parvum oocysts were suspended in aqueous buffer (10 mM Tris-HCl, pH 7.3, 1 mM $MgCl_2$) containing a protease inhibitor cocktail (Pierce Chemical Co., Rockford, Illinois), and total protein extracted by freezing-thawing. Approximately 10^6 oocysts were mixed with an equal volume of SDS-PAGE sample buffer (Laemmli, 1970) either with or without 10% dithiothreitol (DTT), and heated for 1 min in a boiling water bath, followed by centrifugation at 10,000 g for 5 min. The protein supernatants were loaded onto individual wells of a 10% SDS-PAGE and electrophoresed using standard procedures (Laemmli, 1970). The gels were transferred to Immobilon membrane (Millipore) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, California). The membranes were washed briefly with PBS, and then treated with PBS containing 2% non-fat dry milk (PBS-NFDM) to block non-specific binding of antibodies in subsequent steps. Individual lanes of immunoblots were probed for 2 hr at RT with a 1:1,000 dilution (in PBS containing 0.05% Tween 20) of polyclonal antisera specific for rCp33. Control antisera included sera prior to immunization and sera from rabbits immunized with an irrelevant recombinant polyHis protein. Positive control serum reactive with total *C. hominis* oocysts/sporozoite antigen was used to ensure equivalent amounts of *C. parvum* and *C. hominis* were electrophoresed on SDS-PAGE. Immunoblots were then probed for 2 hr at room temperature (RT) with biotinylated anti-rabbit IgG (1:1,000, Sigma) followed by a 1 hr incubation at RT with alkaline-phosphatase-labeled avidin (1:25,000, Sigma). Blots were washed 3 times with PBS-Tween 20 between each step. The immunoblots were developed by the addition of 0.165 mg/ml 5-bromo-4-chloro-3′-indoylphosphate p-toluidine (BCIP) and 0.33 mg/ml nitro-blue tetrazolium chloride (NBT) (Thermo Scientific, Rockford, Illinois) in alkaline phosphatase buffer (0.1 M Tris, pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$).

Sporozoites of *C. parvum* and *C. hominis* oocysts were excysted using the above excystation protocol, washed with PBS, and then distributed to individual wells of untreated multi-well slides at 10^3 oocysts/well and allowed to air dry. Each well was incubated for 15 min with 20 µl PBS-NFDM, followed by incubation for 2 hr at RT with a 1:250 dilution of anti-Cp33 serum in PBS-Tween 20. Control antisera included sera prior to immunization and sera from rabbits immunized with an irrelevant recombinant polyHis protein, as well as antisera reactive with *C. hominis* oocysts/sporozoites. The oocysts/ sporozoites were then incubated for 1 hr at RT with a 1:50 dilution of FITC-labeled goat anti-rabbit IgG (Sigma) in PBS-Tween20. The wells were washed 3 times between each step by immersing the entire slide in PBS and allowing wells to drain dry. Each well received 5 µl Vectashield (Vector Laboratories, Burlingame, California), overlaid with a coverslip, and examined by epifluorescence microscopy on a Zeiss microscope at ×1,000 magnification. Images were captured using a Zeiss AxioScope camera and AxioVision imaging software. Immunostained parasites were also viewed by using a Zeiss 710 confocal laser scanning confocal microscopy system. For confocal microscopy, *C. parvum* sporozoites were stained with 4′,6-diamidino-2-phenylindole (DAPI) prior to immunolabeling to assist in localizing the nucleus (Campbell et al., 1992). The images were observed using a Zeiss Axio Observer inverted microscope with 100 × 1.4 NA oil immersion Plan Aapochromatic objective. A photomultiplier tube captured in a single-track mode the specimen fluorescence excited by a 488-nm diode laser and emitted fluorescence passing through a MBS 488 beam splitter filter, a pin hole of 33 µm with limits set between 490 and 535 nm for detection of FITC-conjugated goat anti-rabbit IgG antibodies. Zeiss ZenTM 2010 software was used to obtain the images.

Immunostaining of recombinant cgd8-5370 protein expressed in *E. coli* as a poly-His fusion protein with anti-His antibody identified a unique 26 kDa protein (data not shown). This observed Mr was about 3 kDa larger than expected based on the cgd8-5370 amino acid sequence (~18 kDa) and the polyHis fusion partner (~5 kDa). Antisera prepared against recombinant cgd8-5370 identified a 33 kDa native *C. parvum* sporozoite/oocysts protein under non-reducing (–DTT) and reducing (+DTT) SDS-PAGE (Fig. 1). Anti-Cp33 sera did not identify the 33 kDa homologue in *C. hominis* (Fig. 1). The anti-Cp33 sera showed weak reactivity to 30 and 55 kDa proteins in both *C. parvum* and *C. hominis* oocyst/sporozoite proteins under reducing conditions (Fig. 1). However, control sera (pre-immunization and against an irrelevant polyHis recombinant antigen) also showed reactivity with these 2 proteins. The size of the immunoreactive 33 kDa native protein was considerably higher than the predicted 17.5 kDa protein based on the encoded amino acid sequence. Searching the Cp33 amino acid sequence for sites that may be N- or O-glycosylated (http://www.cbs.dtu.dk/services/NetNGlyc/, http://www.cbs.dtu.dk/services/NetOGlyc/) identified 2 potential N-glycosylation sites and 11 potential O-glycosylation sites. This may explain in part the discrepancy between expected and observed relative mass of Cp33.

Immunofluorescence staining of excysted *C. parvum* sporozoites with anti-recombinant Cp33 sera localized the native protein on sporozoites

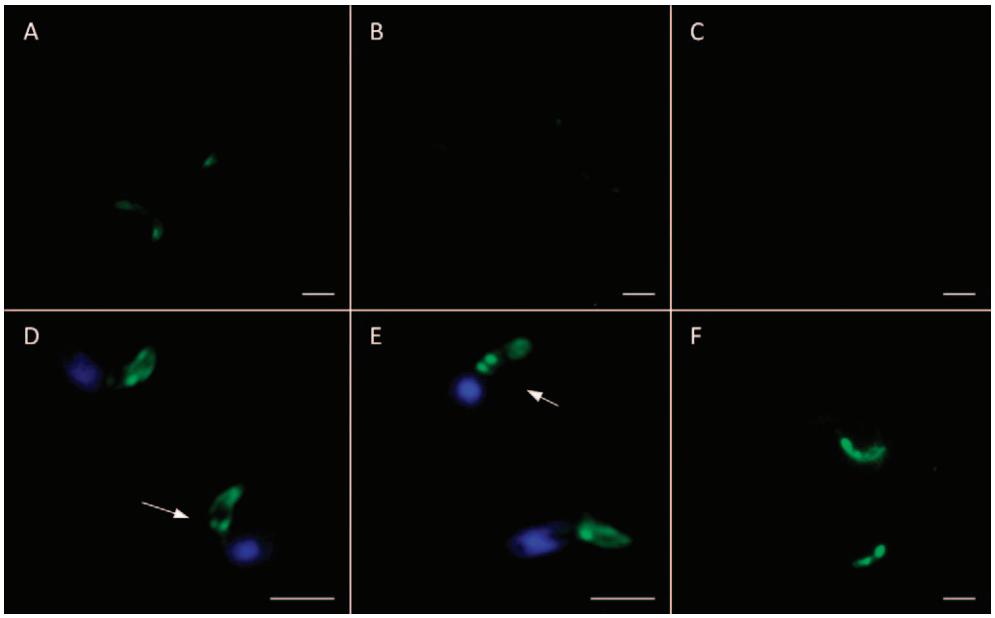

FIGURE 2. Immunofluorescence staining of air-dried *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts and sporozoites with antisera specific for recombinant Cp33. (**A, C, D, E**) Antisera against recombinant Cp33 antigen. (**B**) Antisera against irrelevant recombinant polyHis protein. (**F**) Antisera against total *C. hominis* protein. (A, B, D, E) *C. parvum* sporozoites; (C, F) *C. hominis* sporozoites. Bar = 2 μm.

(Fig. 2A). Binding of *C. parvum* sporozoites was negligible with antisera against an irrelevant recombinant polyHis protein (Fig. 2B) or with pre-immunization sera (data not shown). Likewise, anti-recombinant Cp33 sera failed to recognize *C. hominis* sporozoites (Fig. 2C), although antisera to total *C. hominis* protein displayed strong recognition of *C. hominis* sporozoites (Fig. 2D). Confocal laser scanning microscopy of *C. parvum* sporozoites after DAPI staining revealed binding of anti-Cp33 primarily in the anterior end of the parasite and forward of the nucleus, which others have shown to localize in the posterior of *C. parvum* (Tetley et al., 1998; Riordan et al., 2003; Ctrnacta et al., 2006) (Fig. 2E, F). An occasional sporozoite displayed intense localized staining posterior to the apical end of the parasite (Fig. 2E, F, arrowheads). In an attempt to further localize the Cp33 protein within the parasite, live *C. parvum* sporozoites were stained in suspension with anti-Cp33 sera, followed by FITC-anti-rabbit IgG similar to the procedure for air-dried sporozoites. No staining of live *C. parvum* sporozoites was observed, suggesting that Cp33 is not on the parasite surface (data not shown).

One explanation for the lack of cross-reactivity of anti-Cp33 serum between *C. parvum* and *C. hominis* sporozoites is the relatively high dissimilarity of the nucleotide (Fig. 3A) and primary amino acid (Fig. 3B) sequences. Only 64% of amino acid residues are conserved between *C. parvum* and *C. hominis* Cp33 homologues. Aligning *C. parvum* and *C.*

A

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            ************* ********* ****************************************** ** ********************** *********  ********** ********************** **** **** *
C. parvum   ATGCTTTTGAATAAGAACTCTTCGTCAAAGTCATTCTGTTTTACTTTTTTAGTTTTGTTTTATCTGTGTGTTTATTTATATTTATACGAAGAGAAAATCGTCGTCAGAACTTATACGATTTCTCTAGTTAAGCTACAGACTTTACCTAAC 150
C. hominis  ATGCTTTTGAATATGAACTCTTCATCAAAGTCATTCTGTTTTACTTTTTTAGTTTTGTTTTATCTGCGTATTTATTTATATTTATACGAAGAAAAAATCGTCTCCAGAACTTATGCGATTTCTCTAGTTAAGCTACAAACTTCACCTGAT 150
            1.......10........20........30........40........50........60........70........80........90.......100.......110.......120.......130.......140.......150

             ****** ******** **   ** *** ************ * ************ ***** * * **  *  ** ** * *** *** ***** * **** ** ************ *  ********** **************  *
C. parvum   GTAGACGAAATTGATCAGAGAAGTACTGGTAGTAATCCTGAACGGAAACATGATGACGGAGATCAGACTAAAGCTGAAGATACTACTGGTGTGGTGGGCGGAACCTATGGTCCTGTAAGATGTCGATGCCCTAGTGCCCCCGGTTTCCAG 300
C. hominis  ATAGACGGAATTGATCGGAACGGTGCTGTTAGTAATCCTGAGCAGAAACATGATGAGGGAGACCGGGCTGGAAATGGAGGTGCTATTGGCGTGGTTGACGGACCCCATGGTCCTGTAAAACATCGATGCCCTGGTGCCCCCGGTTTCGTG 300
            .......160.......170.......180.......190.......200.......210.......220.......230.......240.......250.......260.......270.......280.......290.......300

            ************* ****** *    ** ********** *** *** ***  *  *  ** ************ ****** ****    **** *  * ** ****** ****** ******* *****   ********** ******
C. parvum   ATCCTGTCGGTTGTAGACATGGGACCAACCAAAAATAAAGAAGGGAATGGTAATGAAGGACATCGCCTTCGTAATAGACACAAGAGGGGGTCAGGAAAGAATAGAAAAAGGAATTCCTCCGGGCTTAAACCACACACTGATTCGGGAAAT 450
C. hominis  ATCCTGTCGGTTGAAGACATTGACAAAAACAAAAATAAAAAAGAGAAAGGTCGTAGAAAACGTCGCCTTCGTAAAAGACACGAGAGT---TCAGAACGGGATGGAAAAAAGAATTCTTCCGGGCGTAAACTGGACACTGATTCCGGAAAT 447
            .......310.......320.......330.......340.......350.......360.......370.......380.......390.......400.......410.......420.......430.......440.......450

            **************************
C. parvum   GATGCTGATTGTGAAAGTACTGAGTAG 477
C. hominis  GATGCTGATTGTGAAAGTACTGAGTAG 474
            .......460.......470.......
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B

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C. parvum       1 MLLNKNSSSKSFCFTFLVLFYLCVYLYLYEEKIVVRTYTISLVKLQTLPNVDEIDQRSTG 60
                  MLLN NSSSKSFCFTFLVLFYL +YLYLYEEKIV RTY ISLVKLQT P++D ID+
C. hominis      1 MLLNMNSSSKSFCFTFLVLFYLRIYLYLYEEKIVSRTYAISLVKLQTSPDIDGIDRNGAV 60

C. parvum      61 SNPERKHDDGDQTKAEDTTGVVGGTYGPVRCRYPSAPGFQILSVVDMGPTKNKEGNGNEG 120
                  SNPE+KHD+GD+       GVV G +GPV+ R P APGF ILSV D+   KNK+  G
C. hominis     61 SNPEQKHDEGDRAGNGGAIGVVDGPHGPVKHRCPGAPGFVILSVEDIDKNKNKKEKGRRK 120

C. parvum      121 HRLRNRHKRGSGKNRKRNSSGLKPHTDSGNDADCESTE 158
                   RLR RH+  S ++ K+NSSG K  TDSGNDADCESTE
C. hominis     121 RRLRKRHE-SSERDGKKNSSGRKLDTDSGNDADCESTE 157
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C

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Amino Acid Sequence     MLLNKNSSSK SFCFTFLVLF YLCVYLYLYE EKIVVRTYTI SLVKLQTLPN    50
Predicted Topology      OOOOOOOooo oHHHHHHHHH HHHHHHHHHi iiiiiiiiii iiiiIIIIII

Amino Acid Sequence     VDEIDQRSTG SNPERKHDDG DQTKAEDTTG VVGGTYGPVR CRYPSAPGFQ   100
Predicted Topology      IIIIIIIIII IIIIIIIIII IIIIIIIIII IIIIIIIIII IIIIIIIIII

Amino Acid Sequence     ILSVVDMGPT KNKEGNGNEG HRLRNRHKRG SGKNRKRNSS GLKPHTDSGN   150
Predicted Topology      IIIIIIIIII IIIIIIIIII IIIIIIIIII IIIIIIIIII IIIIIIIIII

Amino Acid Sequence     DADCESTE  158
Predicted Topology      IIIIIIII
```

FIGURE 3. Alignment of cgd8-5370 *Cryptosporidium parvum* and *Cryptosporidium hominis* DNA (**A**) and predicted amino acid (**B**) sequences. (**C**) Predicted topology of Cp33 protein (o, outer portion; h, transmembrane helix; I, i, internal region).

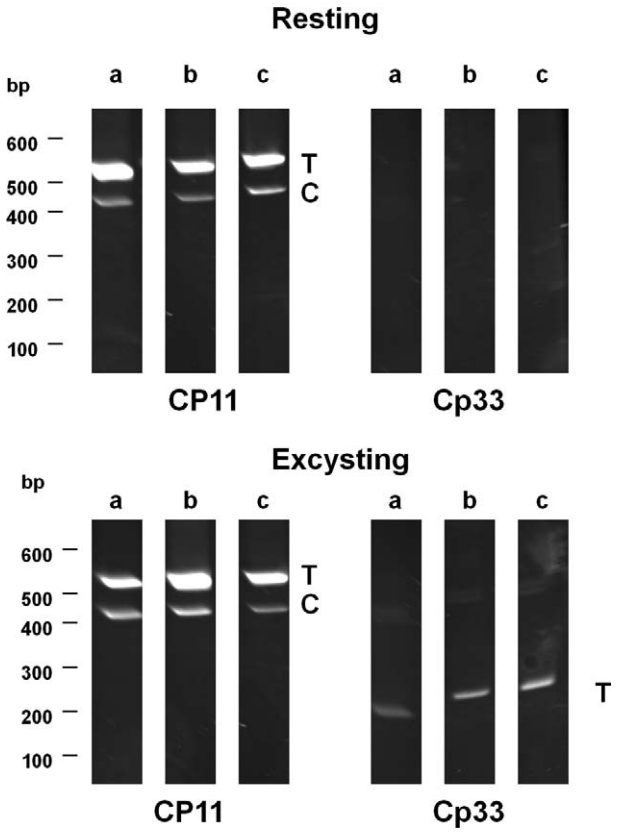

FIGURE 4. RT-PCR analysis of Cp33 mRNA in resting (R) and excysting (E) *Cryptosporidium parvum* oocysts. CP11 T, 570 bp target amplification product; CP11 C, 410 bp competitor (internal standard) amplification product; Cp33 T, 196 bp target amplification product; a–c, 3 replicate PCR or RT-PCR amplifications.

hominis Cp33 sequences showed high sequence identity in the N- and C-terminal ends of the protein, but much lower identity over the internal 100 amino acid (residues 50–147, Fig. 3B). The N-terminal region (AA nos. 11–30) was found by sequence analysis (http://www.cbs.dtu.dk/services/TMHMM-2.0/) to contain a signal peptide, and the overall topology of the protein is consistent with a short extracellular domain, a membrane-spanning region, and a large cytoplasmic domain (http://www.enzim.hu/hmmtop/ server/hmmtop.cgi) (Fig. 3C). These predictions are consistent with the observed cytoplasmic IFA staining of *C. parvum* sporozoites and also suggest that the immunogenic regions of the protein are contained within the variable cytoplasmic domain. Others using monoclonal antibodies have found a lack of cross-reactivity between *C. hominis* and *C. parvum* P23 and GP900 antigens in immunoblotting assays (Sturbaum et al., 2008). It remains unknown whether the non-cross-reactive epitopes of Cp33, P23, or GP900 are involved in determining host range.

Cp33 may play a role in preparing *C. parvum* sporozoites for invading host cells. RT-PCR analysis of *C. parvum* oocysts before or during excystation revealed transcription only during excystation (Fig. 4). Consistent with this observation, cgd8-5370 expressed sequence tags shown in the cryptoDB database (Heiges et al., 2006) originate exclusively from sporozoites. However, there is evidence for low-level expression of cgd8-5370 in *C. parvum* during in vitro development (Mauzy et al., 2012). Localization of Cp33 to the sporozoite cytoplasm and our inability to achieve staining of live *C. parvum* sporozoites with anti-Cp33 sera suggest that the protein is not involved in cell attachment. BLAST-N and BLAST-P searching of the GenBank database failed to identify any significant similarities with non-*Cryptosporidium* sequences.

In conclusion, this study demonstrates that *C. parvum* and *C. hominis* differ phenotypically with respect to the 33 kDa homologue. The role that Cp33 plays in *C. parvum* development remains unknown, although Cp33 transcription appears to be temporally restricted to excysting sporozoites. The protein localizes in the cytoplasm and is concentrated primarily at the anterior end of *C. parvum* sporozoites. The lack of anti-Cp33 sera binding to *C. hominis* sporozoites may reflect a role of this protein in host specificity.

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LITERATURE CITED

ABRAHAMSEN, M. S., T. J. TEMPLETON, S. ENOMOTO, J. E. ABRAHANTE, G. ZHU, C. A. LANCTO, M. DENG, C. LIU, G. WIDMER, S. TZIPORI, ET AL. 2004. Complete genome sequence of the apicomplexan, *Cryptosporidium parvum*. Science **304:** 441–445.

BOUZID, M., P. R. HUNTER, V. MCDONALD, K. ELWIN, R. M. CHALMERS, AND K. M. TYLER. 2013. A new heterogeneous family of telomerically encoded *Cryptosporidium* proteins. Evolutionary Applications **6:** 207–217.

CAMPBELL, A. T., L. J. ROBERTSON, AND H. V. SMITH. 1992. Viability of Cryptosporidium parvum oocysts: Correlation of in vitro excystation with inclusion or exclusion of fluorogenic vital dyes. Applied and Environmental Microbiology **58:** 3488–3493.

COLLINET-ADLER, S., AND H. D. WARD. 2010. Cryptosporidiosis: Environmental, therapeutic and preventive challenges. European Journal of Clinical Microbiology and Infectious Diseases **29:** 927–935.

CTRNACTA, V., J. G. AULT, F. STEJSKAL, J. S. KEITHLY, L. PUTIGNANI, A. TAIT, H. V. SMITH, D. HORNER, J. TOVAR, L. TETLEY, ET AL. 2006. Localization of pyruvate: NADP+ oxidoreductase in sporozoites of *Cryptosporidium parvum*. Journal of Eukaryotic Microbiology **53:** 225–231.

FAYER, R. 2004. *Cryptosporidium*: A water-borne zoonotic parasite. Veterinary Parasitology **126:** 37–56.

GE, G., L. COWEN, X. FENG, AND G. WIDMER. 2008. Protein coding gene nucleotide substitution pattern in the apicomplexan protozoa *Cryptosporidium parvum* and *Cryptosporidium hominis*. Comparative and Functional Genomics. **2008:** Article ID 879023; doi: 10.1155/2008/879023.

HANAHAN, D. 1983. Studies on the transformation of *Escherichia coli* with plasmids. Journal of Molecular Biology **166:** 557–580.

HEIGES, M., H. WANG, E. ROBINSON, C. AURRECOECHEA, X. GAO, N. KALUSKAR, P. RHODES, S. WANG, C. Z. HE, Y. SU, ET AL. 2006. CryptoDB: A *Cryptosporidium* bioinformatics resource update. Nucleic Acids Research **34:** D419–D422.

JENKINS, M. C., C. O'BRIEN, K. MISKA, R. S. SCHWARZ, J. KARNS, M. SANTIN, AND R. FAYER. 2011. Gene expression during excystation of *Cryptosporidium parvum* oocysts. Parasitology Research **109:** 509–513.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685.

MAUZY, M. J., S. ENOMOTO, C. A. LANCTO, M. S. ABRAHAMSEN, AND M. S. RUTHERFORD. 2012. The *Cryptosporidium parvum* transcriptome during in vitro development. PLoS ONE **7:** e31715. doi: 10.1371/journal.pone.0031715.

RIDER, S. D., AND G. ZHU. 2010. *Cryptosporidium*: Genomic and biochemical features. Experimental Parasitology **124:** 2–9.

RIORDAN, C. E., J. G. AULT, S. G. LANGRETH, AND J. S. KEITHLY. 2003. *Cryptosporidium parvum* Cpn60 targets a relict organelle. Current Genetics **44:** 138–147.

ROBINSON, G., AND R. M. CHALMERS. 2012. Assessment of polymorphic genetic markers for multi-locus typing of *Cryptosporidium parvum* and *Cryptosporidium hominis*. Experimental Parasitology **132:** 200–215.

SAMBROOK, J., E. F. FRITSCH, AND T. MANIATIS. 1989. Molecular cloning, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1626 p.

STURBAUM, G. D., B. H. JOST, AND C. R. STERLING. 2003. Nucleotide changes within three *Cryptosporidium parvum* surface protein

encoding genes differentiate genotype I from genotype II isolates. Molecular and Biochemical Parasitology **128:** 87–90.

———, D. A. Schaefer, B. H. Jost, C. R. Sterling, and M. W. Riggs. 2008. Antigenic differences within the *Cryptosporidium hominis* and *Cryptosporidium parvum* surface proteins P23 and GP900 defined by monoclonal antibody reactivity. Molecular and Biochemical Parasitology **159:** 138–141.

Tanriverdi, S., and G. Widmer. 2006. Differential evolution of repetitive sequences in *Cryptosporidium parvum* and *Cryptosporidium hominis*. Infection Genetics and Evolution **6:** 113–122.

Tetley, L., S. M. Brown, V. Mcdonald, and G. H. Coombs. 1998. Ultrastructural analysis of the sporozoite of *Cryptosporidium parvum*. Microbiology **144:** 3249–3255.

Widmer, G., and Y. Lee. 2010. Comparison of single- and multilocus genetic diversity in the protozoan parasites *Cryptosporidium parvum* and *C. hominis*. Applied and Environmental Microbiology **76:** 6639–6644.

———, ———, P. Hunt, A. Martinelli, M. Tolkoff, and K. Bodi. 2012. Comparative genome analysis of two *Cryptosporidium parvum* isolates with different host range. Infection Genetics and Evolution **12:** 1213–1221.

———, and S. Sullivan. 2012. Genomics and population biology of *Cryptosporidium* species. Parasite Immunology **34:** 61–71.

Xu, P., G. Widmer, Y. Wang, L. S. Ozaki, J. M. Alves, M. G. Serrano, D. Puiu, P. Manque, D. Akiyoshi, A. J. Mackey, et al. 2004. The genome of *Cryptosporidium hominis*. Nature **431:** 1107–1112.

Zhang, H., F. Guo, H. Zhou, and G. Zhu. 2012. Transcriptome analysis reveals unique metabolic features in the *Cryptosporidium parvum* oocysts associated with environmental survival and stresses. BMC Genomics **13:** 647.